

Detection of Low Numbers of Bacterial Cells in Soils and Sediments by Polymerase Chain Reaction

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Polymerase chain reaction was used to amplify the low copy number of two 16S ribosomal gene fragments from soil and sediment extracts. Total DNA for polymerase chain reaction was extracted from 1 g of seeded or unseeded samples by a rapid freeze-and-thaw method. Amplified DNA fragments can be detected in DNA fractions isolated from seeded soil containing less than 3 *Escherichia coli* cells and from seeded sediments containing less than 10 cells. This research demonstrated that coupling polymerase chain reaction to direct DNA extraction improves sensitivity by 1 and 2 orders of magnitude for sediments and soils, respectively. This technique could become a powerful tool for genetic ecology studies.

Polymerase chain reaction (PCR) technology has proven to be a revolutionary method which gives scientists the great advantage of generating a large number of target DNA sequences of interest from trace amounts of DNA material. Since its first introduction in 1985 (13), PCR has already become a widespread technique in numerous research laboratories. In addition to pure research (8, 10, 11) and medical (7, 9, 12) applications, PCR technology has recently been applied to water (1, 2, 6), sediment (16), and food samples (19). In the present study, we have coupled a rapid direct DNA extraction method (18) with the PCR to detect a low cell density or a low gene copy number of bacterial cells in soil and sediments. This protocol will benefit the study of monitoring intentionally or accidentally released, genetically engineered microorganisms and the investigation of detoxification or biodegradation genes in the environment.

Soil and sediment samples were collected from a manufactured gas site in southern California (SC) and from a settling pond in Oak Ridge, Tenn. (ORT), respectively. The ORT sediments possessing high cation-exchange capacity (CEC; 34 meq/100 g) contained 51% water, and the SC soil, characterized by low CEC (9 meq/100 g), consisted of 10% water. All soil and sediment samples were quantified as wet weight throughout the experiments. Total DNA was extracted from 1 g of soil or sediment samples and suspended in 200 μ l of sterile TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) by a rapid method, as described previously (18). The test samples included sterile seeded or unseeded and nonsterile seeded or unseeded soil and sediments. The organism used for seeding was an *Escherichia coli* strain, strain 35346 (ECOR27), purchased from the American Type Culture Collection, Rockville, Md. ECOR27 was grown at 37°C in nutrient broth (Difco, Detroit, Mich.) to the late exponential phase and harvested by centrifugation at 3,000 \times g for 10 min at 4°C. The resulting pellet was washed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄) before seeding, and the resting cells were enumerated on nutrient agar plates. The

sterile environmental samples were obtained by autoclaving at 121°C for 30 min.

PCR was used to amplify two target fragments on the *E. coli* 16S rRNA gene. The reaction mixture contained 200 μ M each dATP, dCTP, dGTP, and dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, and 0.3 μ M concentrations of each primer (20-mer RW01 [5'-AACTGGAGGAAGGTGGGGAT-3'] and 19-mer DG74 [5'-AGGAGGTGATCCAACCGCA-3'] or 20-mer RW01 and 15-mer DG74 [5'-ACGGCGGTGTGTAC]). At 80°C, 10 μ l of reaction mixture containing 2.5 U of low DNA AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added to each 90- μ l reaction mixture with an appropriate dilution (0 to 50,000 copies of 16S ribosome gene) of purified *E. coli* genomic DNA template. For environmental samples, 1 μ l of undiluted SC DNA extract or 1 μ l of 10⁻²-fold-diluted ORT DNA extract was used as the template in the PCR. Each reaction mixture (100 μ l) was heated to 95°C for 2 min, which was followed by 40 PCR cycles (one cycle is 1.5 min at 95°C for denaturation and 1 min at 62°C for annealing and extension) and a 7-min final extension at 62°C, using a programmable DNA thermocycler (Perkin-Elmer Cetus). The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with 1 μ g of ethidium bromide per ml.

For verification of the amplified PCR product, an internal 20-mer probe with the sequence 5'-TACGTTCCCGGGCCTGTAC-3' was used. The oligonucleotide probe was end labelled with [γ -³²P]ATP (specific activity = 3,000 Ci/mmol; New England Nuclear Products) by a T4 polynucleotide kinase (BioLabs, Beverly, Mass.), as described by Sambrook et al. (14). The amplified DNA fragments were transferred to a nylon membrane (GeneScreen Plus; New England Nuclear Products, Boston, Mass.) by Southern blotting (15) or to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by slot blotting (5). The hybridization and washes were carried out under high-stringency conditions, as described previously (17).

Figure 1 shows a PCR-amplified, 371-bp DNA fragment on the *E. coli* 16S rRNA gene, using primers RW01 and DG74. The PCR was able to amplify this fragment from less than 3 fg of total genomic DNA, i.e., from less than 5 copies (1 ag) of 16S rRNA gene. An estimated 10⁸-fold increase in DNA quantity was obtained after amplification. Due to the use of "hot-start" PCR (4), the formation of primer-dimer was

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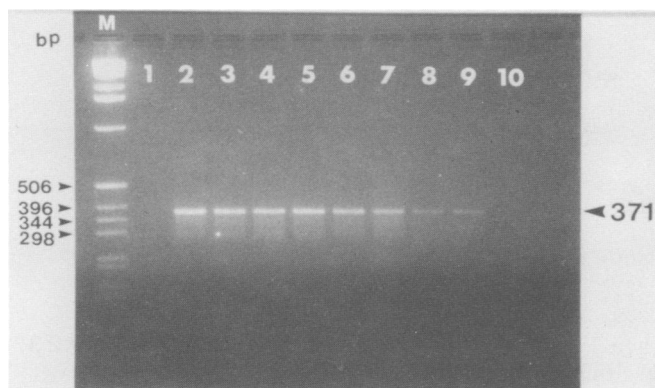


FIG. 1. Sensitivity of detection by PCR on a 16S rRNA gene fragment of *E. coli*. Lanes: M, DNA 1-kb ladder marker (1 μ g); 1, no genomic DNA; 2, 30 ng of *E. coli* genomic DNA (5×10^7 copies of 16S rRNA gene); 3, 3 ng (5×10^6 copies); 4, 300 pg (5×10^5 copies); 5, 30 pg (5×10^4 copies); 6, 3 pg (5×10^3 copies); 7, 300 fg (5×10^2 copies); 8, 30 fg (5×10^1 copies); 9, 3 fg (5 copies); 10, 300 ag (0.5 copies).

minimized and no nonspecific priming was found during the reaction. As evidenced by the negative control (lane 1, Fig. 1), the results also confirmed that the batches of low DNA AmpliTaq used were free of *E. coli* DNA, or at least *E. coli* DNA contamination was not detected by the amplification.

The sterile SC soils were seeded with various densities of ECOR27 ranging from 0 to 5.0×10^8 cells per g. Following the direct DNA extraction from 1 g of each seeded soil, a 1- μ l aliquot from 200 μ l of crude DNA was used as a template for hot-start PCR. Two sets of primers were used in the reaction, resulting in one 237-bp fragment being amplified by primers RW01 and 15-mer and one 371-bp fragment being amplified by primers RW01 and DG74, as shown in Fig. 2A. The sensitivity of detection was determined as 500

cells per g on the ethidium-stained gel (Fig. 2A) compared with 5,000 cells per g by the gene probe method (18). However, since only 1/200 of the seeded (500 cells) soil extract was used for PCR, this method was actually able to detect less than 3 *E. coli* cells. Assuming that there are 7 copies of 16S rRNA genes in each *E. coli* cell (3), the detection limit was 21 copies of the target sequences. Figure 2B illustrates the hybridization signals from the same gel after Southern transfer by using the earlier-described internal oligonucleotide probe. Lower quantities of the amplified 371-bp fragment were obtained than of the 237-bp fragment, indicating better amplification efficiency for the shorter target sequence (Fig. 2A and B). Reducing the denaturing time to 1 min and increasing the annealing time to 1.5 min might improve the amplification of the larger DNA fragment. Because low DNA AmpliTaq polymerase was used throughout the experiment, no DNA contaminant from the enzyme itself was found in the negative control (lanes 1 and 10, Fig. 2). For the nonsterile seeded soil, the detection limit was also similar to that found in sterile seeded soil. However, a weakly positive amplification target sequence was observed in unseeded soil. This could be due to homologous 16S rRNA genes from natural heterotrophic bacteria (data not shown), because the heterotrophic bacterial plate count from the indigenous microbial population was determined to be $2.5 \pm 0.5 \times 10^4$ CFU/g on plate count agar (Difco). The soil tested negative for the presence of *E. coli* or coliforms by using Colilert (Access Analytical, Branford, Conn.). The sensitivity of PCR on DNA extracted from environmental samples decreased compared with that on purified genomic DNA. This could be attributed to humic substances or other interfering compounds present in the soil or sediments.

The humic substances obtained from the direct DNA extraction of sterile high-CEC sediments (ORT sediments) were found to inhibit the AmpliTaq polymerase. Figure 3 and Table 1 show the effects of humic-acid-like substances extracted from ORT sediments on PCR. When template

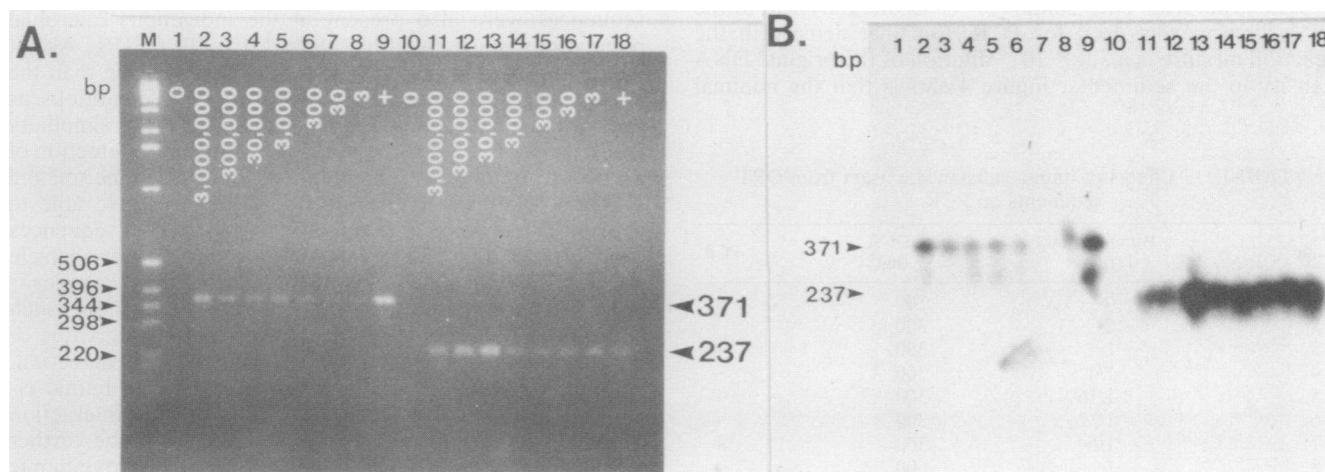


FIG. 2. Sensitivity of detection by PCR on crude DNA extracted from sterile SC soil seeded with various cell densities of ECOR27 cells. (A) Ethidium bromide-stained agarose gel; (B) hybridization signals against an internal oligonucleotide probe after Southern transfer of the former gel. Lanes: M, DNA 1-kb ladder marker (0.5 μ g); 1 and 10, unseeded; 2 and 11, seeded with 5.0×10^8 cells per g (2.5×10^6 cells); 3 and 12, 5.0×10^7 cells per g (2.5×10^5 cells); 4 and 13, 5.0×10^6 cells per g (2.5×10^4 cells); 5 and 14, 5.0×10^5 cells per g (2.5×10^3 cells); 6 and 15, 5.0×10^4 cells per g (2.5×10^2 cells); 7 and 16, 5.0×10^3 cells per g (2.5×10^1 cells); 8 and 17, 5.0×10^2 cells per g (2.5 cells); 9 and 18, ECOR27-positive control (30 ng of genomic DNA). The cell number from each DNA extract used for PCR is indicated in parentheses. Lanes 1 to 9 show the PCR products (371 bp) amplified by primers RW01 and DG74, and lanes 10 to 18 represent the short amplified fragments (237 bp), using primers RW01 and a 15-mer.



FIG. 3. Effects of humic substances extracted from sterile ORT sediments on PCR. (a) Hybridization signals of each sample corresponding to each lane in the agarose gel in part b; (b) ethidium bromide-stained gel. The treatment of each sample for each lane in part b is shown in Table 1.

DNA was not a limiting factor, the inhibition was attenuated by simple dilution of the existing humic substances. The enzyme produced detectable PCR product when the ORT humic extract was diluted 1/32-fold. As little as 1 μ l of undiluted humic-acid-like extract from high-CEC sediments is sufficient to completely inhibit PCR regardless of the amount of DNA present in the 100- μ l reaction mixture, indicating that inhibition is possibly from humic interaction with the enzyme itself or from primer annealing. The results from slot blot hybridization match the data from the ethidium bromide-stained gel (Fig. 3a and b).

In a separate experiment, the MIC of commercially available humic acids on low DNA AmpliTaq polymerase (Aldrich Chemical Co., Milwaukee, Wis.) was determined to be less than 10 ng per 100- μ l reaction mixture. Therefore, to measure the sensitivity of PCR on DNA extracts from high-CEC ORT sediments, a 10^{-2} -fold dilution of crude DNA extract from sterile seeded samples was used. The sterile sediments were seeded with ECOR27 ranging from 0 to 2.0×10^8 CFU/g. Because the total volume for crude DNA extract from each sample is 200 μ l and only 1 μ l of the 10^{-2} dilution was used for PCR, the final density in the reaction mixture is a 5.0×10^{-5} dilution of the original DNA density in the sediments. Figure 4 shows that the minimal

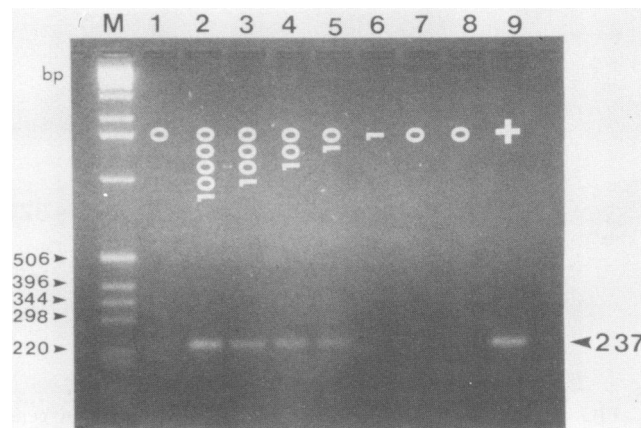


FIG. 4. Sensitivity of detection by PCR on crude DNA extracted from sterile ORT sediments seeded with various cell densities of ECOR27 cells. Lanes: M, DNA 1-kb ladder marker (1 μ g); 1, unseeded; 2, seeded with 2.0×10^8 cells per g (10^4 cells); 3, 2.0×10^7 cells per g (10^3 cells); 4, 2.0×10^6 cells per g (10^2 cells); 5, 2.0×10^5 cells per g (10^1 cells); 6, 2.0×10^4 cells per g (1 cell); 7, 2.0×10^3 cells per g (0 cells); 8, 2.0×10^2 cells per g (0 cells); 9, ECOR27-positive control (30 ng of genomic DNA). The cell number from each DNA extract used for PCR is indicated in parentheses.

cell number for detection of a positive target sequence was 10. Thus, the original sample contained 2.0×10^5 cells per g of sediment. However, no positive hybridization signal against crude DNA extracted from 2.0×10^8 cells per g of sediment (total DNA, 1.5 μ g/g) was found when the 32 P-labeled oligonucleotide probe (20-mer) was used (data not shown). This also suggests that the poor sensitivity of detection for the unamplified total DNA could be due to the small size of the oligonucleotide probe, as previous work with the same sediment showed a sensitivity level of 5,000 cells per g when a 1-kb probe was used (18).

The two desired target sequences were amplified from nonsterile seeded sediments. This suggests that these two sequences were also present in the indigenous microbial populations represented by $3.9 \pm 2.0 \times 10^4$ CFU/g. Again, the 237-bp fragment manifests better amplification than the 371-bp fragment and is attributable to PCR conditions as opposed to environmental sample constraints. For simplicity and sensitivity, PCR is the method of choice for detection of a low copy number and small target sequences in the soil and sediment samples. In the present study, we were able to apply PCR techniques to amplify specific target sequences from low-cell-density soil and sediments. The major obstacle in using PCR on environmental soil and sediment samples is the presence of humic substances or other components such as iron, etc., that can inhibit the polymerase activities or binding of primers and reduce the sensitivity of detection. Although the dilution method can attenuate the humic effects, it also reduces the detection limit. The interaction between humic substances and PCR needs to be further investigated. A good separation procedure to remove humic substances from contaminated DNA without reducing the yields is necessary for increasing sensitivity in PCR.

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TABLE 1. Effects of humic substance extract from ORT sediments on PCR

Lane ^a	Humic extract (μ l) ^b	DNA (ng) ^b	PCR ^c
1	0	300	+
2	2	300	—
3	1	300	—
4	1/4	300	—
5	1/16	300	—
6	1/32	300	+
7	1/64	300	+
8	1	600	—
9	1	300	—
10	1	30	—
11	1	3	—
12	1	0.3	—
13	1	0.03	—

^a Lanes correspond to lanes labeled in Fig. 3.

^b Per 100- μ l reaction mixture.

^c +, positive; —, negative.

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